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THE METABOLIC FATE OF ISOVALERATE, A GROWTH FACTOR FOR RUMINOCOCCUS FLAVEFACIENS

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Based on both cultural counts (1) and direct microscopic observations (2) as well as the in vitro rate of cellulose digestion, it is probable that bacteria belonging to the genus Ruminococcus are among the more important ruminal cellulose digesters. Representative strains of this genus have shown a nutritional requirement for certain of the branched-chain volatile acids normally found in rumen fluid (3, 4).

Bryant and Doetsch (5) had earlier shown that Bacteroides succinogenes, also an important cellulose digester, required volatile fatty acids and more recently other strains including non-cellulolytic bacteria have shown a similar requirement (6, 7). Bentley et al. (8) and MacLeod and Murray (9) have demonstrated that volatile fatty acids stimulate cellulose digestion by the mixed rumen flora.

To obtain information on the metabolic function of these unique growth factors we have determined the fate of labeled carbon after culture of R. flavefaciens, strain C-94, in the presence of isovalerate-1-C^{14} (10) and isovalerate-3-C^{14}. Data shown in table 1 indicate that most of the C^{14} was incorporated into cell protein and lipid. Leucine was the only radioactive amino acid detected in both experiments. Since the carboxyl carbon of the 5 carbon acid was incorporated into leucine, the biosynthetic mechanism apparently differs from that demonstrated in other microorganisms, (11, 12). That the requirement for isovalerate was not due to an absolute inability to utilize exogenous leucine was shown in another experiment in which cells incorporated labeled leucine into cellular protein. However, the organism had a very limited ability to utilize exogenous amino acids including leucine.

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TABLE 1.--Distribution of radioactivity after culture of R. flavefaciens in medium containing (C^{14}) isovalerate

Distribution	isovalerate-1-C^{14} (counts/min/ml)	isovalerate-3-C^{14} (counts/min/ml)
Whole culture	199,000	45,000
Cells	47,000	2,240
Cellular fraction		
Lipid	20,250	570
Nucleic acid	2,500	50
Protein	22,360	1,040

Paper chromatographic analysis of the lipid fraction of cells grown in isovalerate-1-C^{14} showed that there was no detectable C^{14} present as unchanged isovalerate or other short-chain fatty acids nor as α-ketoisocaproate or similar keto acids. Reverse phase paper chromatography using the method of Buchanan (13) demonstrated that the bulk of the C^{14} in the saponified lipid fraction migrated at a rate similar to that expected for a 15-carbon saturated fatty acid.

Methyl esters of the fatty acids were separated by gas-liquid chromatography using a stationary phase of 40 percent Reoplex 400 in 60 to 80 mesh celite. Table 2 shows the relative proportions of methyl esters determined by measuring the area under peaks that were identified by comparing retention times relative to known standard straight-chain compounds. Tentative identification of branched-chain methyl esters was made by correlating with published retention times of known branched-chain fatty acid esters (14) and with chromatograms showing the branched acids in butterfat.

TABLE 2.--The fatty acid composition of total hexane-soluble lipids extracted from R. flavefaciens strain C-94

Fatty acid	Percentage	Fatty acid	Percentage
C_{12}	1.4	C_{16}	23.0
C_{14}-branched	1.4	C_{16}-mono-unsaturated plus C_{17}	7.0
C_{14}	5.4	C_{18}	10.3
C_{15}-branched	27.4	C_{18}-mono-unsaturated	15.7
C_{15}	2.3	plus traces of unidentified acids	
C_{16}-branched	6.2		

Figure 1 shows the distribution of C^{14} in fractions collected during gas chromatography of a sample from which the unsaturated methyl esters had been removed by bromination. Fractions were collected by trapping the vapor eluate from the column in teflon tubes containing glass wool saturated with hexane. The peak corresponding with a branched-chain 15-carbon fatty acid contained 74 percent of the total radioactivity collected. The other fraction with appreciable radioactivity was that containing methyl esters of 17 carbon acids where 13 percent of the total C^{14} was detected.

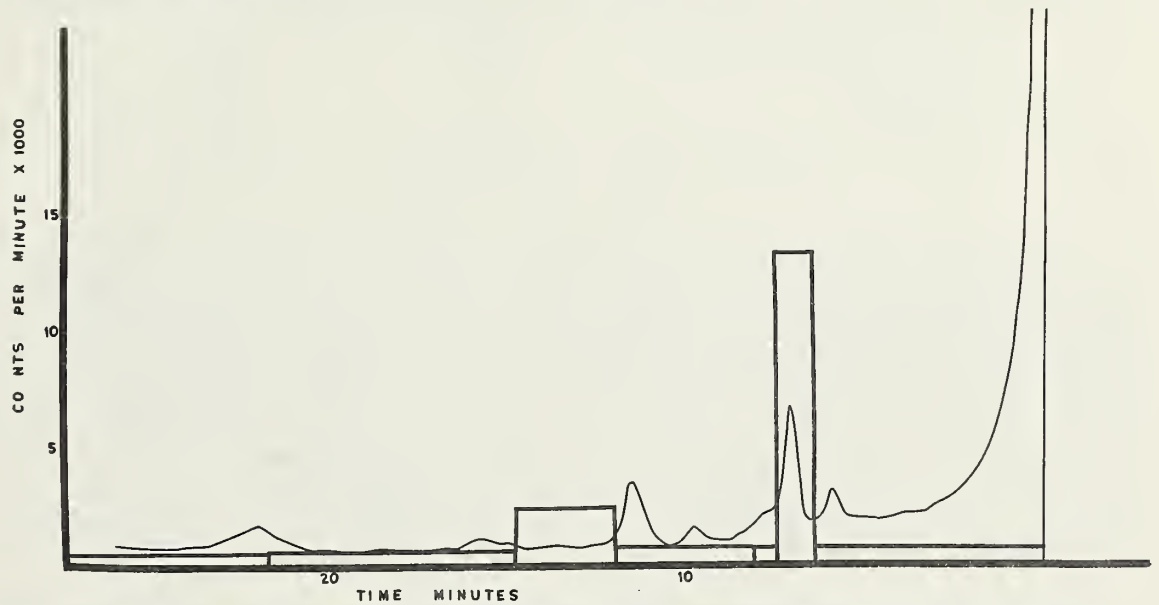

Figure 1.--Radioactivity in fractions (designated by bars) collected during gas chromatography of brominated methyl esters.

Preliminary data showing class separation of the lipid material on a silicic acid column is shown in table 3. Much of the radioactivity was recovered in the phospholipid fraction and most of that not in this fraction was extractable from hexane with Na_2CO_3 and thus was presumably present in free fatty acids. Thirteen percent of the C^{14} in the phospholipid fraction was present in monocarbonyl compounds which were freed when treated with weak acid. This suggests attachment to glycerol through a $\alpha\beta$-unsaturated ether linkage as shown to occur in plasmologens. The labeled carbonyl compounds have not yet been identified but adsorption spectrophotometry and column chromatography (15) indicate the presence of 13 to 15 carbon aldehydes. Thus, it appears that a significant portion of the cellular lipid is present as plasmologen. To our knowledge, plasmologens have not been demonstrated in bacteria but it may be that they are in part responsible for the feulgen postive substance present in the cytoplasmic membrane of many bacteria (16).

TABLE 3.--Distribution of C^{14} in the lipid fraction of R. flavefaciens cultured in isovalerate-1-C^{14} as shown by chromatography on a silicic acid column

Fraction	Solvent	Percent of total counts recovered	
		Whole fraction	Extracted with Na_2CO_3
1	Chloroform	12.1	3.9
2	Chloroform-methanol (98:2)	27.5	24.0
3	Methanol	60.4	----

The high proportion of branched 15-carbon fatty acids as compared with branched-chain even numbered fatty acids may be a reflection of the ratio of isovalerate to isobutyrate in the culture medium. The medium used was similar to the basal medium previously described (3) except tween 80 was deleted and the sodium salts of the following fatty acids were added: acetate (20 μ mole per ml.), isovalerate-1-C^{14} (0.375 μ mole per ml.) and isobutyrate (0.075 μ mole per ml.). It will be interesting to compare the fatty acid composition of cells cultured under these conditions with those grown in other combinations of volatile fatty acids or in rumen fluid.

Branched-chain 15-carbon fatty acids are among those found in butterfat and ruminant body lipids and branched-chain 15-carbon aldehydes have been found in milk fat (17) and are a major component of ox-spleen and ox-liver plasmologens (18). It is suggested that rumen bacteria, such as the strain studied here, are a significant source of the odd-numbered, branched-chain and other higher fatty acids and aldehydes found in ruminant lipids.

The data of Wegner (19) agree with our results as he has found that with this same strain labeled carbon atoms from isobutyrate-1-C^{14} are incorporated into cellular valine and into 14 and 16-carbon fatty acids.

SUMMARY AND CONCLUSIONS

We have shown that in Ruminococcus flavefaciens, strain C-94, radioactivity from isovalerate-1-C^{14} is incorporated into leucine, a branched-chain 15-carbon fatty acid, a 17-carbon fatty acid, and 13 to 15 carbon aldehydes. While these compounds account for most of the cellular radioactivity, other labeled compounds of considerable

physiological importance but having smaller pool size may be present. The exact reason for the branched-chain volatile fatty acid requirement is not yet clear but it seems probable that they function as sources of the isoprene moiety and are required because the ability to synthesize this has been lost or does not function adequately.

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